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Award Number: W81XWH-06-1-0618

TITLE: Gold Nanoparticle Contrast Agents in Mammography: A Feasibility Study

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REPORT DATE: August 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-08-2007	Annual	15 JUL 2006 - 14 JUL 2007

4. TITLE AND SUBTITLE

Gold Nanoparticle Contrast Agents in Mammography: A Feasibility Study

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0618

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pennsylvania
Philadelphia, PA 19104

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The successful translation of molecular imaging to mammography and digital breast tomosynthesis would allow clinical molecular imaging of the breast. This is a potentially more sensitive approach to early breast cancer detection, especially in women at high risk. Bioconjugated gold (Au) nanoparticle (NP) imaging agents, used in conjunction with digital mammography and breast tomosynthesis, should provide improved lesion conspicuity. We are studying the feasibility of mammographic molecular imaging through in vitro "mock tumor" studies of Herceptin conjugated to Au-NP. Au-NP are exceptionally attenuating at mammographic energies; even very low concentrations are theoretically detectable. The primary aim of this study is to prove that Au-NP can act as a viable mammographic contrast agent. To date, we have synthesized bioconjugated HER2/neu Au-NP. Two breast cancer cell-lines have been established: BT-474 over-expresses HER2, while MDA-MB-468 is HER2 negative. We are currently measuring cellular affinity. In the near future, we will be ready to produce cell pellets that can be imaged by digital mammography and digital breast tomosynthesis to test for adequate contrast enhancement, both conventionally and using dual-energy subtraction methods. These data will be sufficient to determine whether mammography molecular imaging agents are feasible.

15. SUBJECT TERMS
Breast Cancer, X-ray contrast agent, Gold Nanoparticles, Her2/neu

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	14	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. Introduction

Bioconjugated gold (Au) nanoparticle (NP) imaging agents, used in conjunction with digital mammography and breast tomosynthesis, should result in significantly improved lesion conspicuity. Molecular imaging agents targeted to bind to specific ligands have been proposed for many imaging modalities[1]. However, we are unaware of any molecular imaging agents proposed for mammography; the most common breast imaging method.

We propose to study the feasibility of mammographic molecular imaging through *in vitro* "mock tumor" studies of analogues of Herceptin (a monoclonal antibody that binds to HER2/neu) conjugated to Au-NP. Au-NP are exceptionally attenuating at mammographic energies; thus, even very low concentrations are theoretically detectable. We calculate that to achieve a 5% change in attenuation at 17.5 keV (Mo K_α) we would require ten 100μm dia. NP per cell; this would allow the detection of a 5 mm dia. tumor at a clinical mammographic dose (1.8 mGy). Using similar agents for thermoacoustic tomography, Copland[2] achieved significant tissue enhancement with a binding density of 18 NP per cell. The combination of such contrast agents with dual-energy subtraction breast tomosynthesis would allow high-resolution cross-sectional molecular imaging *in vivo* and trivial fusion of functional and anatomic images.

In this grant, we have proposed to demonstrate in proof-of-principle the viability of Au-NP targeted mammographic molecular imaging agents. We will investigate fabrication feasibility and contrast sensitivity of these agents. These data will be used for submission of a grant to develop such imaging agents further.

2. Body

2.1. Research Overview

The research conducted over the past year has been directed towards synthesizing molecular imaging agents for use with emerging breast imaging modalities such as digital breast tomosynthesis (DBT) and breast computed tomography (BCT). Molecular imaging agents use specific biomarkers to target tissues or organs so that they can be easily distinguished from their immediate environment.

We have opted to use gold in the form of spherical nanoparticles as the building block for the imaging agents because of the high x ray attenuation of gold at low mammographic energies. The nanoparticles are to be conjugated with an affibody targeting the epidermal growth factor receptor protein, HER2/neu. Studies have shown that over 90% of all breast cancers overexpress either the HER2/neu gene or surface protein. Breast cancer cell lines are being cultured to investigate the binding and uptake of these nanoparticles, and to measure the x-ray imaging properties. These data will provide the data necessary to determine the potential effectiveness of the conjugated gold nanoparticles as contrast agents.

The research therefore consists of three major subsections:

(i) Synthesis and functionalization of gold nanoparticles

(ii) Growth and culturing of breast cancer cell lines

(iii) Mock tumor studies.

The project is ongoing and the results obtained to-date are presented herewith.

2.2. Results

2.2.1. Synthesis of gold nanoparticles

Gold nanoparticles (AuNP) have been synthesized using a modified Turkevich method[3]. This involves the reduction of a gold precursor in the presence of a surface stabilizing agent. We have used gold (III) chloride as the gold metal precursor and sodium citrate as the reducing agent. The latter serves a dual function as both the reducing agent as well as the surface stabilizing molecule. When certain environmental conditions are met in the synthesis of the AuNP, citrate anions will coat the surface of the AuNP, thus preventing them from aggregating out of the solution. The final size and shape distribution is heavily dependant on the molar ratio between the gold(III) chloride and the sodium citrate. We have used molar ratios of 4:1, 3:1, 2:1 and 1:1 to obtain successively larger sized nanoparticles.

Initially, we used the procedure set forth by Grabar *et al.*[4], to synthesize AuNP with an expected diameter in the range of 13nm using a molar ratio of 4:1 between the gold and the sodium citrate. Briefly, 0.20g of gold (III) chloride trihydrate ($HAuCl_4.3H_2O$) was dissolved with 500ml of triple-distilled water in a 1L round-bottom flask to which a Teflon-coated magnetic stir bar was added. The flask was then placed atop a heating mantle and magnetic stirrer and the gold solution was brought to a boil. A quantity

(0.57g) of sodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$) was then dissolved in 50ml of deionized water and then added to the boiling gold solution. Upon addition of the sodium citrate, the solution rapidly changed color from a pale yellow to a dark burgundy. Heating was continued for an additional 10 minutes after which the hearing mantle was removed and the solution was stirred for an extra 15 minutes. The resulting colloidal solution was allowed to cool overnight after which it was filtered through a 0.2 μm membrane filter.

2.2.2. AuNP diameter measurements

Small angle x ray scattering (SAXS) was used to determine the size and distribution of the AuNP[5]. A small sample of the AuNP was placed in a cuvette, sealed with epoxy, and then placed in the path of an x ray beam of known energy. The intensity of the scattered beam is measured at various scattering angles, and a Guinier plot[5] (logarithm of intensity vs. scattering vector) was obtained. The plot is modeled as a series of functions, such as polynomial and Rayleigh functions, to determine best the radius of the AuNP in solution. A subset of these results is shown in Table 1.

Using a molar ratio of 4:1 between the gold (III) precursor and sodium citrate, we obtained AuNP with a diameter of approximately 14nm

Table 1. Radius of AuNP determined from SAXS

Rayl Radius	Value	Standard Error	Lower Limit	Upper Limit
(in nm)	6.980956	0.661E-02	6.951232	7.01167

2.2.3. AuNP Concentration

Using the extinction coefficients for gold nanoparticles provided in the literature[6], and the absorption spectrum of the AuNP solution in the UV-Vis range (Figure 1), we were able to determine the concentration of the AuNP in solution to be 9nM. The UV-Vis spectra of the AuNP shows a peak in the absorbance at a wavelength of 518 nm. This peak is typical of AuNP in solution due to the excitation of surface plasmons on the gold nanostructure[6].

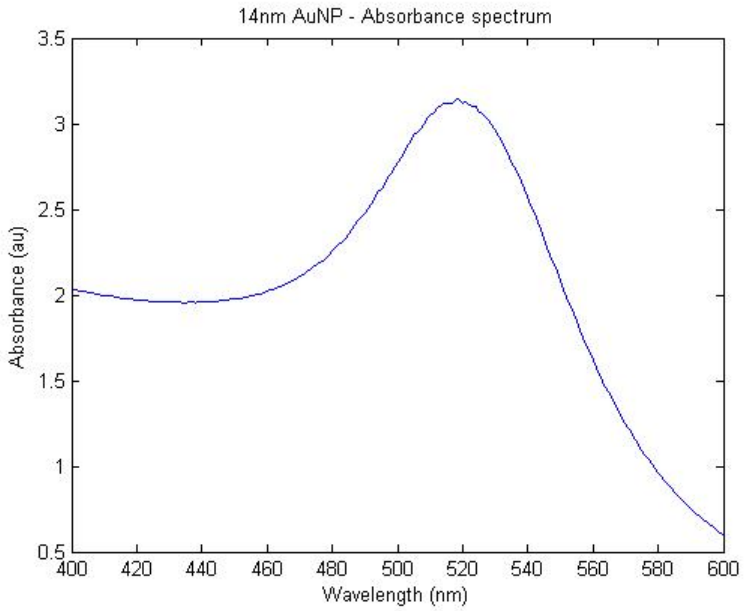

Figure 1. Uv-Vis absorbance spectra of the 14nm AuNP in solution

2.2.4. Stabilization of AuNP with PEG

The citrate-capped AuNP were shown to be unstable in high ionic strength biological media. When 200µl of 14nm AuNP is added to 400µl of phosphate buffered saline (PBS) solution, a buffer used to mimic the extra cellular conditions of the human body, the AuNP were observed to aggregate out of the solution. The UV-Vis spectrum of the aggregated AuNP solution showed a large red-shift and spreading of the absorbance peak as the color of the solution changed from a burgundy-red to a purple blue.

In order to improve their stability, the AuNP will first be conjugated to polyethylene glycol (PEG) peptides in order to increase their stability in salt solutions, as well as enhance their stealth properties *in vivo*. The PEG molecules obtained for the conjugation had an average molecular weight of 5000 Daltons, and were capped with an unreactive methyl group on one end and a thiol (-SH) group on the other.

3ml of 18nm AuNP (2.81 nM) was mixed with 212µl of 0.5µg/µl PEG-thiol solution in a 10ml round-bottom flask, and stirred at room temperature for 1 hour. The solution was then centrifuged at 11,400g for 20 minutes, and the AuNP pellet was redissolved in 2ml of deionized water after the supernatant had been removed. The resulting AuNP-PEG solution was stored at room temperature. The UV-Vis spectrum (Figure 2) of the AuNP-PEG showed a red shift of 3nm in the absorbance peaks between the conjugated and unconjugated AuNP. The shift in the spectrum indicates that the PEG molecules have successfully replaced the citrate anions and bound to the AuNP surface[6].

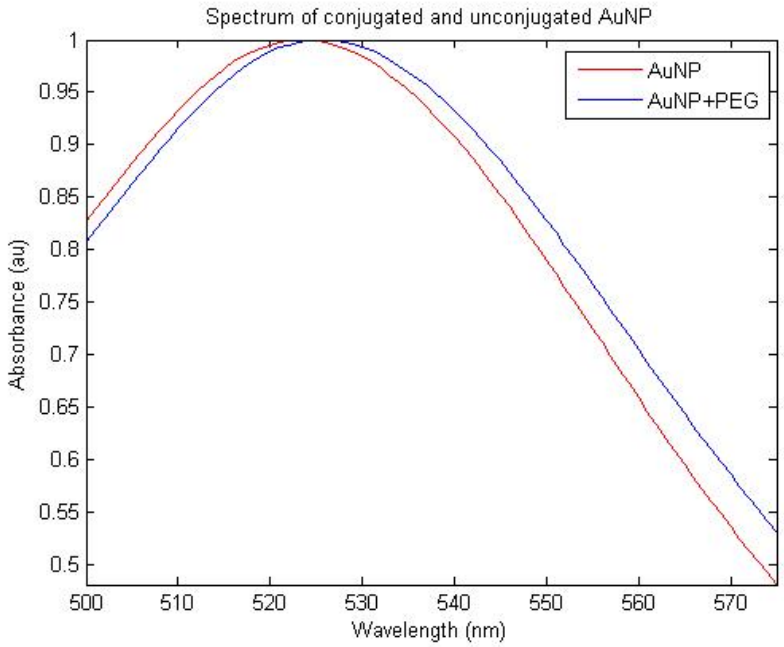

Figure 2. Spectrum of conjugated and unconjugated AuNP

The stability of the conjugated AuNP was testing by adding 200µl of AuNP-PEG solution to 400µl of PBS solution. There have been no observable changes in the color of the solution and the spectrum of the AuNP-PEG was unchanged.

2.2.5. Functionalization of AuNP with PEG and affibodies.

The AuNP have been functionalized with admixtures of PEG and the anti-HER2/neu affibody on the surface of the AuNP. The PEG and affibody are added to the AuNP in series to create a mixed monolayer where the relative surface density of each component depends on its initial concentration. The multi-functionalization of the AuNP is thought to result in improved stability in high ionic-strength biological media as well as specific targeting of tumor cells.

Two forms of the anti-HER2/neu affibody were obtained: an unconjugated affibody in which the C-terminus has been linked to cysteine and a fluorescein-conjugated molecule, in which the C-terminal cysteine has been conjugated to a maleimide-activated fluorescein agent. These affibodies have the same binding sites and binding properties as the native antibodies with the added advantage that they are several times smaller than regular antibodies. The smaller size means that they should exhibit tumor-to-blood ratios that exceed typical values for monoclonal antibodies. The unconjugated molecule will be used for the multi-functionalization of the AuNP whereas the fluorescein-conjugated affibodies will be used in flow cytometry and immuno-staining to compare the expression of the HER2/neu surface protein between the cell lines obtained.

2.2.6. Growth and culturing of breast cancer cell lines

Two breast cancer cell lines, BT-474 and MDA-MB-231 have been obtained. These particular cell lines have been chosen for their differences in the levels of expression of HER2/neu on their cell surface. BT-474 exhibits amplified expression of the protein whereas MDA-MB-231 has a low-to-normal protein expression. Both cell lines have been extensively studied and have been shown to form tumors in nude mice[7,8].

BT-474 cells are grown in RPMI-1640 medium while the MDA-MB-231 cells (Figure 3) are grown in Dulbecco's Modified Eagle's Medium (DMEM). All media must be supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/ streptavidin. Both cell lines are be cultivated in T-75 flasks and incubated at room temperature (37 C) and 5% CO_2. The media must be renewed 2 to 3 times a week, and a subcultivation ratio of 1:2 is being used for both cell lines.

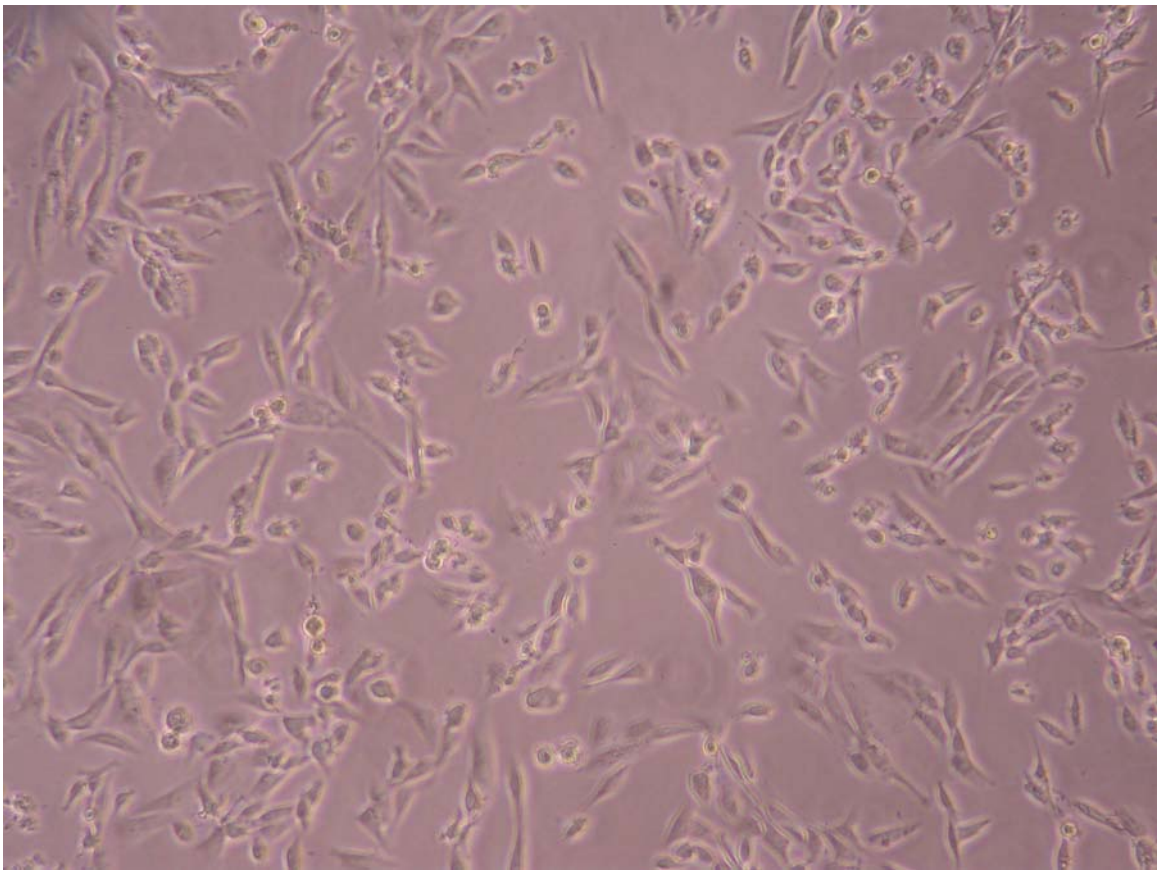

Figure 3. MDA-MB-231 cells

The cell lines will serve two primary purposes. The first is to study the binding and internalization patterns of the affibody-receptor complex in *vitro*. The second (beyond the scope of the current grant) is to form tumors in nude mice to determine the effectiveness and uptake rates of the AuNP *in vivo*.

In order to study the internalization of the affibody-receptor complex, the cell lines will be incubated with a solution of the fluorescein-labeled affibody. The cells will then be washed with an acidic buffer (0.2M glycine- 0.15M NaCl, pH 3.0) to remove the cell surface receptor conjugates. The cells will then be irradiated under the necessary wavelength of light to excite the maleimide agent, and then observed for fluorescence. Any fluorescence measured is a result of the internalized affibody-receptor conjugates. Flow cytometry will be used to determine the binding density of the fluorescein affibody for each cell line. The BT-474 and MDA-MB-231 cell lines will be cultivated in flasks for 2 weeks, until a working bank of 10^7 cells is achieved for each cell line.

3. Key Research Accomplishments:

We have successfully synthesized spherical gold nanoparticles with controlled diameters of between 13 and 100 nm. We have successfully conjugated both PEG (an inert control) and admixtures of PEG and anti-HER2/neu affibodies to the gold nanoparticles. We have demonstrated that the conjugated nanoparticles are stable in serum. We have established two working cell lines. We are currently engaged in experiments to measure affinity of the various conjugates to tumor cells which over- and under-express the HER2/neu antigen. X-ray imaging tests will follow shortly

4. Reportable Outcomes:

1) R Karunamuni, AV Popov, H Qiao, S-J Park, ADA Maidment. HER2/neu-targeted Gold Nanoparticles Contrast Agents for Mammography and Tomosynthesis. Accepted as a poster to *DOD Era of Hope*, Baltimore MD, June 2008.
2) The CDMRP FY08 Predoctoral Traineeship grant "HER2-Targeted Gold Nanoparticle Contrast Agent for Digital Breast Tomosynthesis and Computed Tomography" (BC083334 – R Karunamuni, PI) was recommended for funding on May 19, 2008.

5. Conclusions:

We have successfully been able to produce bioconjugated gold nanoparticles. Molecular imaging is widely seen as the future of medical imaging. However, methods developed to date are only suitable *in vitro* or in laboratory animals. The successful translation of molecular imaging to mammography and digital breast tomosynthesis would allow clinical molecular imaging of the breast. This is a potentially more sensitive approach to early breast cancer detection, especially in women at high risk.

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7. Appendices:

None.